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β-Cyclodextrin derivatives that inhibit anthrax lethal toxin

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Abstract—Recently, we demonstrated that simultaneous blocking of bacterial growth by antibiotics and inhibition of anthrax toxin action with antibodies against protective antigen were beneficial for the treatment of anthrax. The present study examined the hypothesis that blocking the pore formed by protective antigen can inhibit the action of anthrax toxin. The potential inhibitors were chosen by a structure-based design using β-cyclodextrin as the starting molecule. Several β-cyclodextrin derivatives were evaluated for their ability to protect RAW 264.7 cells from the action of anthrax lethal toxin. Per-substituted aminoalkyl derivatives displayed inhibitory activity and were protective against anthrax lethal toxin action at low micromolar concentrations. These results provide the basis for a structure-based drug discovery program, with the goal of identifying new drug candidates for anthrax treatment.

1. Introduction

Bacillus anthracis is one of the most dangerous potential biological weapons. Currently, there is no effective treatment for inhalational anthrax, beyond the administration of antibiotics shortly after exposure.[1] Time delay reduces the effectiveness of antibiotic treatment. Therefore, there is need for new, safe, and efficient treatment to supplement intervention with antibiotics. Major factors playing a role in anthrax infection are the cytotoxic effect of anthrax toxin, and bacteremia leading to oxygen and nutritional substance deprivation, as well as the accumulation of various bacterial and host toxic products, leading to eventual organ failure and death.[2] This research was based on the premise that simultaneous blocking of bacterial growth by antibiotics and inhibition of anthrax toxin action would be beneficial for the treatment of anthrax.[3–5] The two anthrax toxins are formed by three proteins: protective antigen (PA) that either combines with lethal factor (LF) to form lethal toxin (LeTx) or with edema factor (EF) to form edema toxin (EdTx). LF and EF are enzymes targeting substrates within the cytosol, and PA facilitates their transport across the cell membrane forming a heptameric pore.[6–8] Recently, we demonstrated that treatment of mice infected with *B. anthracis* with a combination of the antibiotic ciprofloxacin and partially purified antibodies against anthrax protective antigen drastically increased survival rates in comparison with antibiotic treatment alone.[5] Although promising, antibodies are less attractive as potential drugs in comparison with low molecular weight compounds, which offer potentially better penetration through membranes and are not sensitive to proteases.

The present study examined the hypothesis that compounds designed to block the pore formed by PA can inhibit the action of anthrax toxin. PA assembles into a ring-shaped heptamer with a mostly negatively charged lumen.[9,10] The information available on the three-dimensional structure of the PA pore permits a structure-based drug design. As a starting point for the development of high affinity ligands for the PA pore, we employed β-cyclodextrin (β-CD), a cyclic heptamer of D-glucose having a hydrophobic cavity. In common with the PA-pore, β-cyclodextrin has a sevenfold symmetry. The outside diameter of β-CD—15.3 Å[11]—is of the same order as the diameter of the PA channel lumen, which is 20–35 Å, according to X-ray data,[9] and about 12 Å at its narrowest point, according to

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the measurement of current flow through the channel.[12] It was also demonstrated that β-cyclodextrin can partially block ion conductance through a similar heptameric pore formed by staphylococcal α-toxin.[13] Cyclodextrins are widely used as pharmaceutical agents to enhance solubility, bioavailability, and stability of drug molecules because they can encapsulate organic molecules.[14] Methods for selective modifications of cyclodextrins have been developed and offer excellent opportunities for the synthesis of appropriate derivatives.[15] Presently, several derivatives of β-cyclodextrin are being prepared and evaluated for anti-toxin activity.

2. Results and discussion

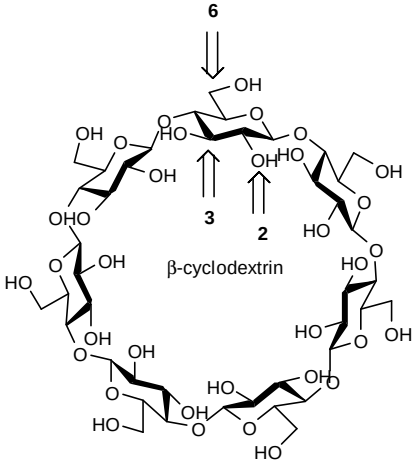
The cyclodextrins α-CD, β-CD, and γ-CD are naturally occurring cyclomaltooligosaccharides containing six, seven, or eight α-(1,4)-D-glucopyranosyl rings.[16] The cyclic array of saccharides produces a hydrophobic cavity, generally described as a truncated cone. The primary alcohols are located around the narrow rim and the secondary alcohols around the wider rim, as shown in Figure 1. The use of CD derivatives as molecular scaffolds is of interest since the primary (C-6) and secondary (C-2 and C-3) hydroxyl groups may be used as points of functionalization.[15]

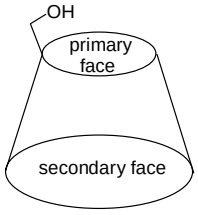
Monofacially functionalized β-CD derivatives substituted on the face containing the primary alcohol with seven positively charged pendant groups represent potentially interesting molecules to block the function of anthrax lethal toxin.

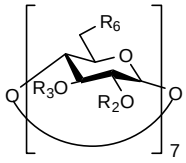
Based on the considerations described above, hepta-6-aminoalkyl derivatives of β-cyclodextrin were chosen for modification. β-CDs substituted with positively charged groups seemed promising because the lumen of the PA pore is negatively charged. Conceivably, the positively charged groups might also alter the effective local pH inside the lumen, inhibiting the conformational change required for the formation of the functional transmembrane channel.[17]

2.1. Chemistry

An important reaction reported by Defaye and co-workers enables the symmetric derivatization of cyclodextrins on the face containing the primary OH groups.[18] This reaction allows the selective







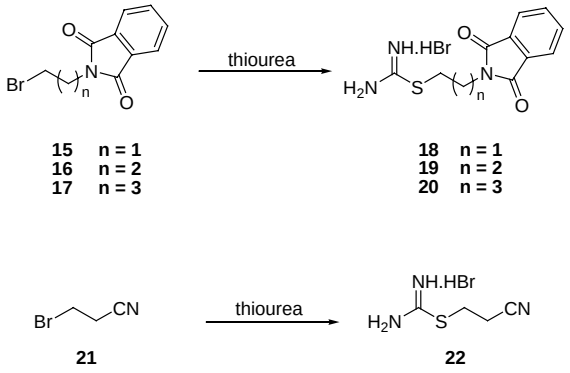
	R_2	R_3	R_6
1	H	H	$SCH_2CH_2NH_2$ (AmEtβCD)
2	H	H	$SCH_2CH_2CH_2NH_2$ (AmPrβCD)
3	H	H	$SCH_2CH_2CH_2CH_2NH_2$ (AmBuβCD)
4	H	H	NH_2 (AmβCD)
5	H	H	I
6	H	H	N_3
7	H	H	SH
8	H	H	OSO_3Na
9	Ac	Ac	OSO_3Na
10	Me	Me	OSO_3Na
11	Me	SO_3Na	OSO_3Na
12	$(CH_2CH_2O)_n$ (n = 2, 3)	H	NH_2
13	$(CH_2CH_2O)_n$ (n = 2, 3)	H	OH
14	H	H	H

Figure 1. Cyclodextrins employed for study.

replacement of all primary hydroxyl groups of a cyclodextrin by iodine atoms. Thus, treatment of β-cyclodextrin with I_2 and PPh_3 in DMF at 70 °C afforded the heptaiodide **5** in 76% yield (Scheme 1). The reaction was performed using a modification to the original protocol, as reported by Stoddart and coworkers.[19] The heptaiodide **5** was treated with NaN_3 in DMF to give the heptaazide **6** in quantitative yield. Heptaazide **6** was subjected to reduction using PPh_3, followed by treatment with ammonia to give the per-6-amino-β-cyclodextrin **4**.

However, the introduction of an alkylamino group at the primary position of β-cyclodextrins proved to be a challenge. The direct alkylation of per-iodo-β-cyclodextrin with an alcoholate nucleophile (derived from an azidoalkanol, for example) would pose some problems since the basic alcoholate may induce elimination or intramolecular substitutions. Some studies have shown that nucleophilic displacement of iodide anions from per-6-iodo-β-cyclodextrins, employing poor nucleophiles or elevated temperatures, favors the intramolecular substitution reaction, resulting in the formation of 3,6-anhydro-D-glucopyranose residues within the structure of per-6-iodo-β-cyclodextrin.[20]

To facilitate the isolation of pure compounds by chromatography and to assure that the products obtained were symmetrically substituted, per-6-iodo-β-cyclodextrin **5** was acetylated using Ac_2O and pyridine in the presence of a catalytic amount of DMAP to give the acylated product **23** in 81% yield (Scheme 2).[18b] Compound **23** reacted smoothly in DMF in the presence of Cs_2CO_3 with thioureido derivatives **18**, **19**, or **20** (obtained from the corresponding *N*-phthalimido-bromoalkyl precursors by treatment with thiourea in EtOH (Scheme 3))[21] to give the desired products **24**, **25**, and **26**. Finally, simultaneous removal of the

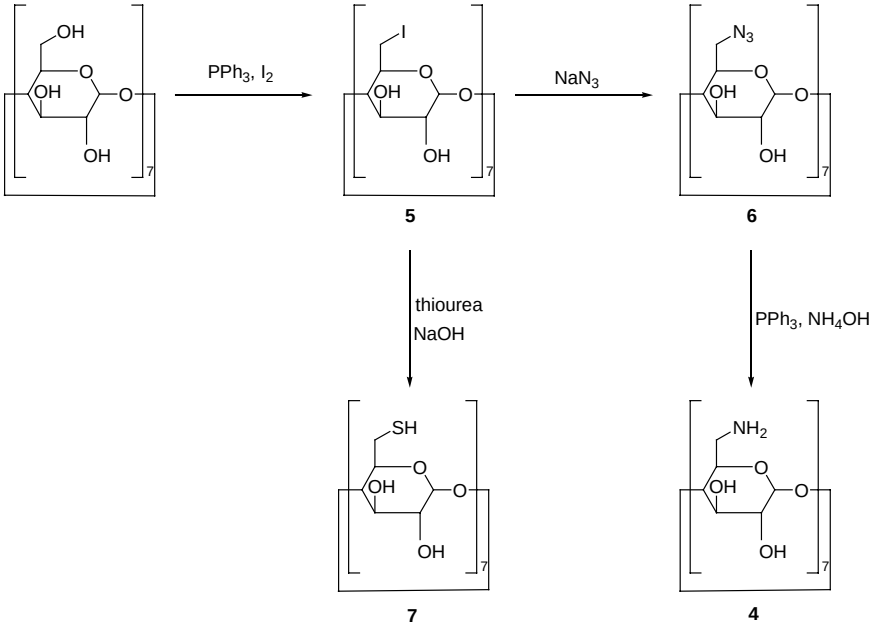


Scheme 2.

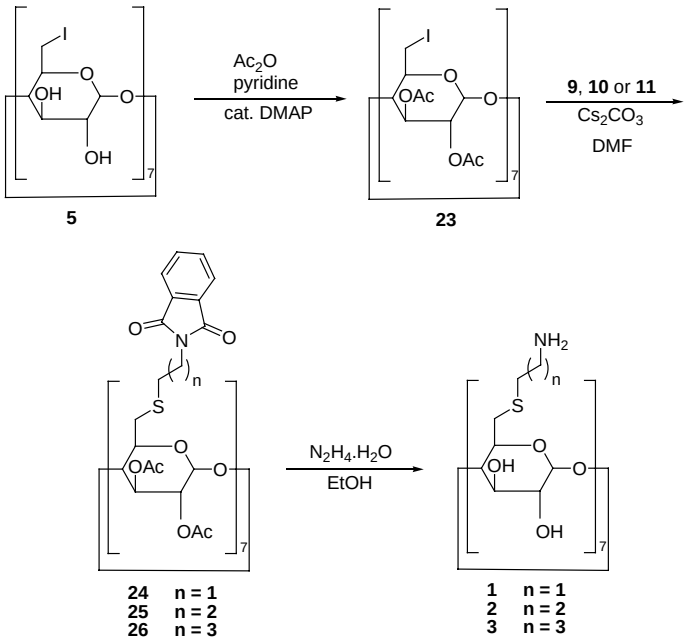
phthalimide and the acetate groups using a large excess of hydrazine monohydrate in aqueous EtOH gave the desired products **18**–**20**, isolated as the hydrochlorides. The structures and symmetrical substitution have been confirmed by mass spectrometry and ^{13}C NMR spectroscopy (Scheme 4).

2.2. Cytotoxicity tests

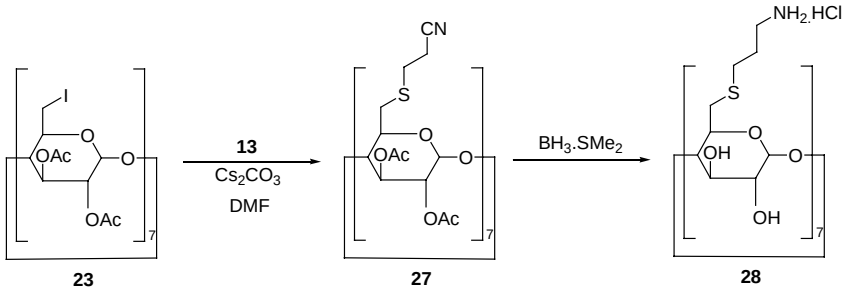
The four hepta-6-aminoalkyl derivatives of β-cyclodextrin, suggested by our structure-based design (**1**–**4**), were synthesized. These and several other β-cyclodextrin derivatives (Fig. 1) were tested for their ability to inhibit the cytotoxic effect of LeTx on mouse macrophage-like RAW 264.7 cells. Strikingly, only the four aminoalkyl derivatives envisaged as inhibitors of LeTx action actually displayed LeTx inhibitory activity, and they were protective against LeTx action at low micromolar concentrations (Fig. 2). These experiments also showed that the compounds were not toxic to RAW 264.7 cells up to 50 μM concentration, while they had IC_{50} values as low as 3.3 μM. The rest of the compounds shown in Figure 1



Scheme 1.



Scheme 3.



Scheme 4.

displayed no inhibitory activity at concentrations up to 100 μM.

2.3. Ion conductance studies

One of the aminoalkyl derivatives—AmPrβCD—was tested for its ability to block ion conductance through PA channels reconstituted in planar bilayer lipid membranes. It was demonstrated that the addition of AmPrβCD to the bilayer lipid membrane with multiple PA channels caused a significant step-like decrease in membrane conductance at 3 nM AmPrβCD concentration (E. Nestorovich and S. Bezrukov, personal communication).

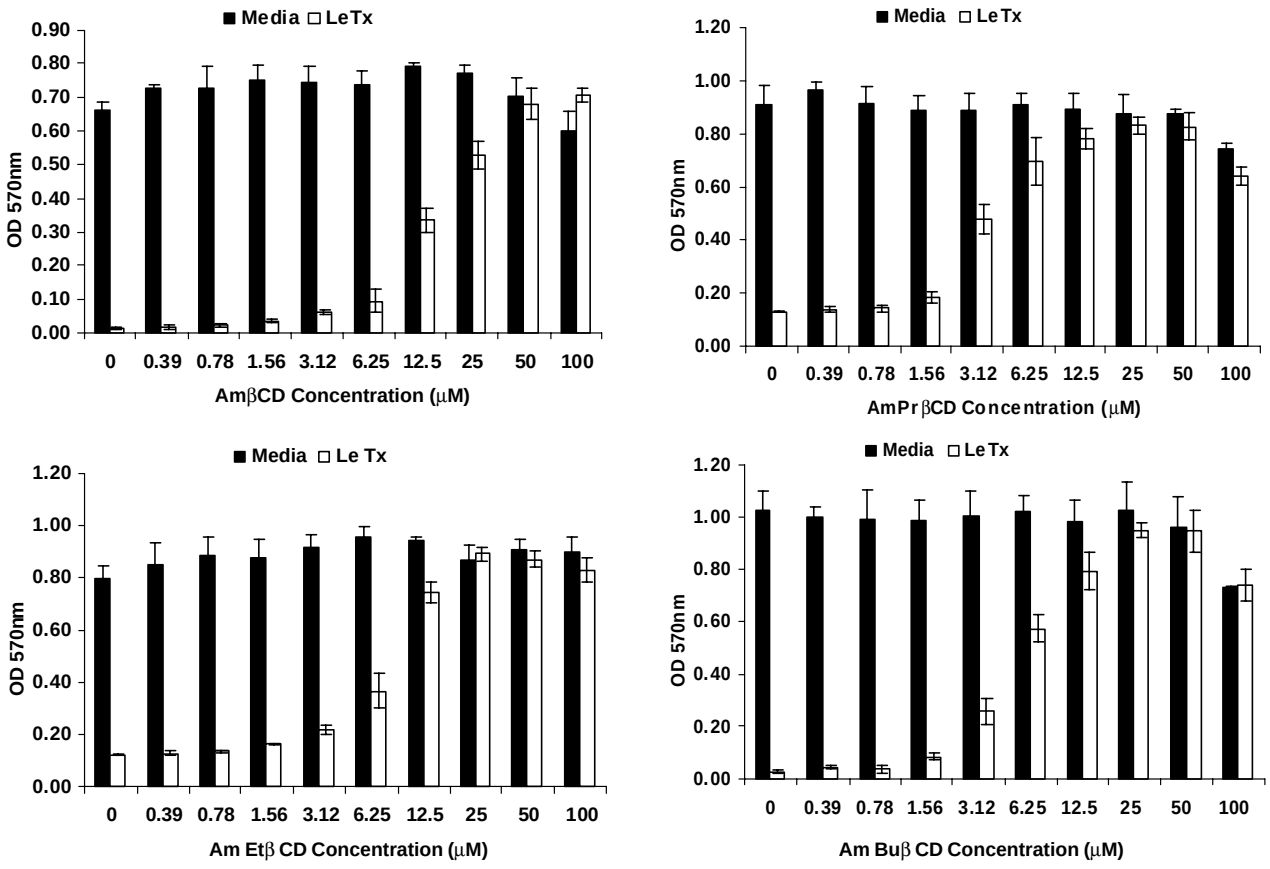
3. Conclusions

The data obtained support the hypothesis that compounds designed to block the pore formed by PA can inhibit the action of anthrax toxin. The results presented provide the basis for a structure-based drug discovery program to identify new drug candidates for anthrax treatment. Per-substituted β-cyclodextrin derivatives can potentially also be used to block other toxins that form heptameric transmembrane channels, such as staphylococcal α-hemolysin. Derivatives of hexameric α-cyclodextrin may also find utility against targets, such as *Helicobacter pylori* VacA toxin or hepatitis C virus p7 protein, which form hexameric channels and are considered to be important virulence factors in the pathogenesis of peptic ulcer disease and HCV infection, respectively. More generally, this can be considered to be a general approach for the discovery of new drugs by blocking pores with molecules having the same dimensions and symmetry as the pores.

4. Experimental

4.1. Chemistry

β-Cyclodextrin derivatives **1**–**7** listed in Figure 1 were synthesized at Pinnacle Pharmaceuticals, Inc. Thio β-cyclodextrin derivatives **8**–**11** were kindly provided by Dr. Gyula Vigh (Texas A&M University, College Station, TX). Compounds **12** and **13** were purchased



IC_{50} :

AmβCD	12.1 ± 3.5 μM
AmEtβCD	9.3 ± 0.8 μM
AmPrβCD	3.3 ± 1.0 μM
AmBuβCD	6.1 ± 2.3 μM

Figure 2. Protection of RAW 264.7 cells from LeTx-induced cell death by β-CD derivatives. RAW 264.7 cells were incubated with different concentrations of the β-CD derivatives with or without LeTx. Each experimental condition was performed in triplicate. Cell viability was determined by MTS colorimetric assay. Error bars represent standard deviations. IC_{50} values are means of three to four independent experiments.

from Cytrea (Dublin, Ireland). β-Cyclodextrin (**14**) was purchased from Sigma Chemicals (St. Louis, MO).

4.1.1. General methods. ^1H NMR and ^{13}C NMR spectra were recorded on a General Electric QE-300 or a Varian 300 spectrometer. Moisture sensitive reactions were conducted under argon in oven-dried glassware. All chemical reagents were purchased from Aldrich Chemicals or Fisher Scientific and used without further purification. Acetonitrile and dichloromethane were distilled from CaH_2. DMF was distilled from CaH_2 under diminished pressure. Triethylamine was distilled from P_2O_5. Analytical thin-layer chromatography was performed on Merck $60F_{254}$ precoated silica gel plates. Visualization was performed by ultraviolet light or by staining with phosphomolybdic acid or sulfuric acid. Flash chromatography was performed using (40–60 μm) silica gel. Melting points were taken with a Mel-Temp melting point apparatus and are uncorrected. Cyclodextrins **4**, **5**, **6** and **7** were prepared according to literature procedures.[18,19,22]

4.1.2. (2-Phthalimidoethyl)isothiouronium hydrobromide (18).[23] A suspension of 3.0 g (11.8 mmol) of *N*-(2-bromoethyl)phthalimide (**15**) and 1.82 g (23.96 mmol) of thiourea in 5.7 mL of absolute EtOH was stirred at reflux for 18 h after which the product had crystallized. The cooled product was collected by filtration, washed with small amounts of cold abs EtOH, and dried in vacuo. Compound **18** was obtained as colorless crystals: yield 4.0 g (100%); mp 243–245 °C; ^1H NMR (DMSO-d_6) δ 3.53 (t, 2H, $J = 5.9$ Hz), 3.91 (t, 2H, $J = 5.9$ Hz), 7.93 (m, 4H), and 9.07 (br s, 3H).

4.1.3. (3-Phthalimidopropyl)isothiouronium hydrobromide (19).[24] A suspension of 3.0 g (11 mmol) of *N*-(3-bromopropyl)phthalimide (**16**) and 1.7 g (22.4 mmol) of thiourea in 5.3 mL of abs EtOH was stirred at reflux for 18 h at which time the product had crystallized. The cooled product was collected by filtration, washed successively with two 10-mL portions of cold abs EtOH and two 10-mL portions of ether, and then dried in vacuo. Compound **19** was obtained as colorless crystals:

yield 3.95 g (100%); mp 236–238 °C (lit.[25] 236–237 °C); ^{1}H NMR (DMSO-d_6) δ 1.97 (m, 2H), 3.22 (t, 2H, J = 6.6 Hz), 3.71 (t, 2H, J = 6.2 Hz), 7.88 (m, 4H), and 9.07 (br s, 3H).

4.1.4. (4-Phthalimidobutyl)isothiouronium hydrobromide (20).[25] A suspension of 1.0 g (3.5 mmol) of *N*-(4-bromobutyl)phthalimide (**17**) and 0.54 g (7.08 mmol) of thiourea in 1.7 mL of abs EtOH was stirred at reflux for 18 h. Upon cooling to room temperature, the syrupy mixture began crystallizing and was treated with 4 mL ether. The mixture was stirred for 15 min and the product was collected by filtration and washed with a small amount of cold EtOH. Compound **20** was obtained as a colorless solid: yield 1.22 g (96%); mp 171–172 °C; ^{1}H NMR (DMSO-d_6) δ 1.70 (m, 4H), 3.18 (t, 2H, J = 6.6 Hz) 3.63 (t, 2H, J = 6.1 Hz), 7.89 (m, 4H), and 9.00 (br s, 3H).

4.1.5. Heptakis (2,3-di-*O*-acetyl-6-deoxy-6-iodo)cyclomaltoheptaose (23).[18b] To a solution of 1.0 g (0.52 mmol) of per-6-iodo-β-cyclodextrin (**5**) in 5 mL of dry pyridine was added 7.5 mL of Ac_2O and 6.5 mg (0.05 mmol) of 4,4-dimethylaminopyridine. The reaction mixture was stirred at 23 °C under argon for 48 h. The reaction was quenched by the addition of 15 mL MeOH and the solvent was concentrated under diminished pressure. Co-evaporation with three 4-mL portions of MeOH and three 4-mL portions of toluene gave a brown residue, which was purified on a silica gel column (20 × 3 cm). Elution with a step gradient of 1:1 → 1:4 hexane–EtOAc gave compound **23** as a colorless foam, which crystallized upon trituration with ether: yield 1.06 g (81%): mp 180–182 °C (lit.[18b] 172–177 °C); ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H), 2.09 (s, 3H), 3.58–3.81 (m, 4H), 4.83 (dd, 1H, J = 9.9, 3.9 Hz), 5.20 (d, 1H, J = 3.6 Hz), and 5.33 (br t, 1H, J = 8.4 Hz); mass spectrum (MALDI), *m*/*z* 2514.9 $[M+Na]^+$, theoretical *m*/*z* 2514.8.

4.1.6. Heptakis [2,3-di-*O*-acetyl-6-deoxy-6-(2-phthalimidoethyl)-thio]cyclomaltoheptaose (24). To a solution of 0.5 g (0.2 mmol) of heptakis (2,3-di-*O*-acetyl-6-deoxy-6-iodo)cyclomaltoheptaose (**23**) and 0.99 g (3.0 mmol) of (2-phthalimidoethyl)isothiouronium hydrobromide (**18**) in 20 mL of dry DMF was added 1.63 g (5.0 mmol) of Cs_2CO_3 and the reaction mixture was stirred at 23 °C under argon for 48 h. The reaction mixture was poured onto 40 g ice and 200 mL of 0.5 N HCl was added. The aqueous layer was extracted with three 50-mL portions of dichloromethane. The combined organic phase was washed successively with 200 mL of 0.5 N HCl and 100 mL of brine, dried ($MgSO_4$), and concentrated under diminished pressure. The residue was purified on a silica gel column (21 × 3 cm); elution with EtOAc afforded compound **24** as a colorless solid: yield 145 mg (23%). An additional 165 mg of **24** was obtained in a slightly impure form; ^{1}H NMR ($CDCl_3$) δ 2.01 (s, 3H), 2.05 (s, 3H), 2.64 (m, 2H), 3.03 (m, 2H), 3.63 (m, 2H), 3.87 (t, 1H, J = 8.4 Hz), 4.15 (m, 1H), 4.80 (m, 1H), 5.10 (br s, 1H), 5.25 (t, 1H, J = 8.7 Hz), 7.62 (m, 2H) and 7.73 (m, 2H); mass spectrum (MALDI), *m*/*z* 3068.8 $[M+Na]^+$, theoretical 3068.7.

4.1.7. Heptakis [2,3-di-*O*-acetyl-6-deoxy-6-(3-phthalimidopropyl)-thio]cyclomaltoheptaose (25). To a solution of 250 mg (0.1 mmol) of heptakis (2,3-di-*O*-acetyl-6-deoxy-6-iodo)cyclomaltoheptaose (**23**) and 472 mg (1.37 mmol) of (3-phthalimidopropyl)isothiouronium hydrobromide (**19**) in 10 mL of dry DMF was added 687 mg (2.11 mmol) Cs_2CO_3 and the reaction mixture was stirred at 23 °C under argon for 68 h. The reaction mixture was poured onto 50 g of ice and 100 mL of 0.5 N HCl was added. The aqueous layer was extracted with three 50-mL portions of dichloromethane. The combined organic phase was washed successively with 100 mL of 0.5 N HCl and 100 mL brine, dried ($MgSO_4$), and concentrated under diminished pressure. The residue was purified on a silica gel column (14 × 3 cm); elution with EtOAc gave compound **25** as a colorless foam: yield 188 mg (59%); ^{1}H NMR ($CDCl_3$) δ 1.91 (m, 2H), 2.02 (s, 3H), 2.05 (s, 3H), 2.60 (m, 2H, J = 12.9, 5.9 Hz), 3.03 (m, 2H), 3.66 (t, 2H, J = 6.9 Hz), 3.84 (t, 1H), 4.12 (m, 1H), 4.80 (dd, 1H, J = 9.7, 3.8 Hz), 5.06 (d, 1H, J = 3.8 Hz), 5.23 (dd, 1H, J = 9.6, 8.3 Hz), 7.58 (dd, 2H, J = 5.4, 3.1 Hz) and 7.70 (dd, 2H, J = 5.5, 3.0 Hz); mass spectrum (MALDI), *m*/*z* 3166.8 $[M+Na]^+$, theoretical 3166.8.

4.1.8. Heptakis [2,3-di-*O*-acetyl-6-deoxy-6-(4-phthalimidobutyl)-thio]cyclomaltoheptaose (26). To a solution of 404 mg (0.16 mmol) of heptakis (2,3-di-*O*-acetyl-6-deoxy-6-iodo)cyclomaltoheptaose (**23**) and 0.87 g (2.4 mmol) of (4-phthalimidobutyl)isothiouronium hydrobromide (**20**) in 16 mL of dry DMF was added 1.32 g (4.04 mmol) Cs_2CO_3 and the reaction mixture was stirred at 23 °C under argon for 48 h. The reaction mixture was poured onto 50 g ice and 200 mL of 0.5 N HCl was added. The aqueous layer was extracted with three 50-mL portions of dichloromethane. The combined organic phase was washed successively with 100 mL of 0.5 N HCl and 100 mL brine, dried ($MgSO_4$), and then concentrated under reduced pressure. The residue was purified on a silica gel column (18 × 3 cm); elution with EtOAc gave compound **26** as a colorless solid: yield 125 mg (24%). An additional 132 mg was obtained in a slightly impure form; ^{1}H NMR ($CDCl_3$) δ 1.61 (m, 2H), 1.73 (m, 2H), 2.02 (s, 3H), 2.06 (s, 3H), 2.65 (m, 2H), 3.03 (m, 2H), 3.63 (m, 2H), 3.88 (m, 1H), 4.15 (m, 1H), 4.80 (dd, 1H, J = 9.8, 3.7 Hz), 5.12 (d, 1H, J = 3.6 Hz), 5.26 (m, 1H), 7.64 (m, 2H), and 7.74 (m, 2H); mass spectrum (MALDI), *m*/*z* 3267.3 $[M+Na]^+$, theoretical 3267.5.

4.1.9. Per-6-(2-aminoethylthio)-β-cyclodextrin (1). A mixture of 100 mg (31.9 μmol) of compound **24** and 1.55 mL (31.9 mmol) of hydrazine monohydrate in 1.5 mL of 1:1 EtOH–H_2O was stirred at 60 °C for 18 h. The solvent was concentrated under diminished pressure to give a solid, that was suspended in 5 mL of 1 N HCl and stirred at 23 °C for 8 h. The insoluble material was filtered and the filtrate was diluted with 25 mL acetone, causing the product to precipitate. The supernatant was removed by centrifugation and the product was washed with four 25-mL portions of acetone and dried in vacuo. The product (**1**) was obtained as a colorless solid: yield 46 mg (89%); mp 180–182 °C

(dec); ^{13}C NMR (DMSO-d_6) δ 102.07, 84.49, 72.51, 72.18, 71.28, 32.70, 29.60; mass spectrum (MALDI), *m*/*z* 1548.8 $[M]^+$, theoretical 1548.9.

4.1.10. Per-6-(3-aminopropylthio)-β-cyclodextrin (2). A mixture of 100 mg (31.4 μmol) of compound **25** and 1.54 mL (31.78 mmol) of hydrazine monohydrate in 1.5 mL of 1:1 EtOH–H_2O was stirred at 60 °C for 16 h. The solvent was concentrated under diminished pressure to give a solid, which was suspended in 5 mL of 1 N HCl and stirred at 23 °C for 4 h. The insoluble material was filtered and the filtrate was diluted with 25 mL acetone, causing the product to precipitate. The supernatant was removed by centrifugation and the product was washed with four 25-mL portions of acetone and dried in vacuo. Compound **2** was obtained as a colorless solid: yield 53 mg (85%); mp 161–163 °C (dec); ^{13}C NMR (DMSO-d_6) δ 26.85, 29.71, 33.03, 37.79, 71.41, 72.23, 72.48, 84.52 and 102.09; mass spectrum (MALDI), *m*/*z* 1668.8 $[M+Na]^+$, theoretical *m*/*z* 1668.6.

4.1.11. Per-6-(4-aminobutylthio)-β-cyclodextrin (3). A mixture of 80 mg (25.3 μmol) of compound **26** and 1.22 mL (25.3 mmol) of hydrazine monohydrate in 1.2 mL of 1:1 EtOH–H_2O was stirred at 60 °C for 24 h. The solvent was concentrated under diminished pressure to give a solid, which was suspended in 5 mL of 1 N HCl and stirred at 23 °C for 4 h. The insoluble material was filtered and the filtrate was diluted with 25 mL acetone, causing the product to precipitate. The supernatant was removed by centrifugation and the product was washed with four 25-mL portions of acetone and dried in vacuo. The product was obtained as a colorless solid: yield 40 mg (94%); mp 172–174 °C (dec); ^{13}C NMR (DMSO-d_6) δ 26.12, 32.15, 32.85, 38.40, 71.49, 72.22, 72.47, 84.43 and 102.05; mass spectrum (MALDI), *m*/*z* 1745.9 $[M+Na]^+$, theoretical *m*/*z* 1745.3.

4.2. Cell-based assays

4.2.1. Cells and cell culture. Recombinant *B. anthracis* lethal factor (rLF) and protective antigen (rPA) were acquired from List Biological Laboratories (Campbell, CA). Murine RAW 264.7 monocyte–macrophage cell line (ATCC TIB-71) was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in phenol-red free Dulbecco's modified Eagle's medium (DMEM, Mediatech, Herndon, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml:100 μg/ml penicillin–streptomycin, 0.1 mM non-essential amino acids, and 0.5 mM of 2-mercaptoethanol at 37 °C in a 5% CO_2 atmosphere. The cells were harvested by gentle scraping with a cell scraper and were then washed once with media. RAW 264.7 cells were plated in 96-well flat-bottomed tissue culture plates from Becton–Dickinson (San Jose, CA) at a concentration of 10^5 cells/well in DMEM mentioned above and incubated overnight at 37 °C in 5% CO_2.

4.2.2. Cytotoxicity neutralization assay. RAW 264.7 cells were pre-incubated with different concentrations of tested compounds in DMEM for 1 h at 37 °C in a 5% CO_2 atmosphere. Then DMEM or LeTx (LF = 32 ng/mL; PA = 500 ng/mL) in the media was added, and the plate was incubated under the same conditions for 4 h. Cell viability was estimated using a MTS kit from Promega (Madison, WI). A μ Quant spectrophotometer from Bio-Tek Instruments (Winooski, VT), was used to make OD_{570} readings.

4.3. Ion conductance studies

Ion conductance experiments were performed according to Montal and Mueller[26] with modifications.[27,28] PA channels were reconstituted in planar lipid membranes formed from DPhPC; the membrane bathing solution contained 0.1 M KCl and 1 mM EDTA at pH 6.6. Ion conductance through PA channels was measured in the presence of AmPrβCD.

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